AD_____

MIPR NUMBER: 94MM4548

TITLE: Role of High-Affinity Oncostatin M Receptor in Prevention of Breast Cancer Cell Growth

PRINCIPAL INVESTIGATOR: Jingwen Liu, Ph.D.

CONTRACTING ORGANIZATION: Veterans Administration Medical Center
Boise, Idaho 83702

REPORT DATE: April 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1997	Annual (1 Apr 96 - 31 Mar 97)

4. TITLE AND SUBTITLE
Role of High-Affinity Oncostatin M Receptor in Prevention of Breast Cancer Cell Growth

5. FUNDING NUMBERS
94MM4548

6. AUTHOR(S)
Jingwen Liu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Veterans Administration Medical Center
Boise, Idaho 83702

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Recently, a novel breast cancer specific gene, designated *BCSG1*, was isolated from human breast cancer cDNA library by the differential cDNA sequencing approach. The mRNA of *BCSG1* is highly expressed in the infiltrating carcinomas but not expressed in the normal or benign breast tissues. We examined the expression of BCSG1 expression in different breast cancer cell lines and investigated the effect of Oncostatin M (OM) on *BCSG1* gene expression in breast cancer cells. A kinetic study showed that treatment of H3922 cells with OM initiated an immediate decrease of *BCSG1* mRNA as early as 30 min. By 4 h, the level of *BCSG1* mRNA was decreased to 70% of control, and by 24 h, the mRNA was completely undetectable. The effect of OM on *BCSG1* was dose-dependent with the maximal suppression at concentration of 1 ng/ml. Results from nuclear run-on analysis and mRNA stability studies indicated that the observed OM-induced down-regulation of *BCSG1* mRNA occurs at the transcriptional level. The effect of OM on BCSG1 gene expression was not shared by other OM related cytokines including IL-6, IL-11, and LIF. These cytokines neither inhibited H3922 cell growth nor BCSG1 transcription. Together, our study suggest that down regulation of BCSG1 expression may be involved in OM-mediated growth inhibition of breast cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	28
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

X Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jing Wen Liu 5/6/97

PI - Signature Date

Table of Contents

A: INTRODUCTION

Human oncostatin M (OM), a product of activated T cells and macrophages, is a 28 kDa glycoprotein that regulates cell growth and differentiation. OM stimulates the growth of normal fibroblasts, normal vascular smooth muscle cells, and myeloma cells. OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including melanoma and lung carcinoma (1-7). The growth inhibitory or growth stimulatory activity exhibited by OM seems to depend on target cell type.

OM signal transduction occurs through two types of heterodimeric receptors (8-10). A shared receptor mediates both OM, LIF and CT-1 signals and is composed of the LIF receptor α subunit and gp130 (9,11). Many overlapping functions of OM and LIF are mediated through this common receptor. However, we and others have demonstrated that a specific OM receptor exists in certain cell types including breast cancer cells. (9,10,12). Therefore, In the grant, we originally proposed to isolate and characterize this OM specific receptor and we predicted that the OM-specific receptor transduces exclusively OM signals and would be consist of gp130 as a binding subunit and a second subunit as an affinity conversion subunit. However, during our initial work to construct a cDNA library and to clone this second subunit, an abstract reporting the isolation of signaling subunit of OM receptor was published in the full of 1994 at the International Cytokine Society Meeting. Bruce Mosley at Immunex reported the cloning of this subunit (13). Therefore, we decided not to waste our resource to continue to clone this subunit, instead to start a collaboration with Immunex to characterize the expression of this newly identified molecule in breast cancer and in normal mammary epithelial cells, and to investigate the functional roles of OM-specific receptor versus the OM/LIF shared receptor in OM mediated growth inhibition, since currently, little is known about how the cellular growth response to OM is controlled at the receptor level, and the molecular mechanism(s) by which OM regulates cell growth remains largely uncharacterized. Unfortunately, although Immunex had agreed to provide the cDNA of OSMRβ for our transfection experiment in the beginning of 1995, their manuscript that describes the cDNA sequence and cloning procedure was delayed for publication until December 1996 (14). Therefore, we were unable to start the experiments as planed in our grant. Recently, we have estabolished the material transfer agreement for the OSMRβ cDNA (April 7, 1997, see attached). As oon as the cDNA becomes avaliable, we will begin to perform the transfection experiments.

In the mean time, we have continued our study to examine the biological functions of OM in breast cancer cells. Previously, we have examined the effects of OM on 10 breast cancer cell lines and on normal human mammary epithelial (HME) cells derived from 4 donors. Our data showed that the cellular proliferation of 7 out of the 10 breast cancer cell lines was inhibited by OM. The 3 cell lines that did not respond to OM treatment lacked OM high affinity binding sites. The growth inhibitory activity of OM is partially due to its functional antagonism of breast cancer mitogens including EGF, EGF-like growth factor, and bFGF and also to its effect on transcriptional suppression of the c-myc protooncogene. In contrast to the selective effect of OM on breast cancer cells, all of the HME cells examined were growth-inhibited by OM. These results led us to examine and to compare the expression levels of the two OM receptors, the OM-specific receptor composed of gp130 and OSMRβ and the OM/LIF shared receptor (LIFR), in HME and breast cancer cells. The flow cytometry analysis utilizing anti-gp130 and anti-OSMRβ

showed that the expression levels of the OM-specific receptor are much higher in HME cells than in breast cancer cells. The OM-nonresponsive breast cancer cells express gp130, the ligand binding subunit, but the OSMRβ, the signaling subunit is undetectable. This suggests that the expression of the signaling component of the OM-specific receptor is dysregulated in breast cancer cells, and this may account for the loss of responsiveness to OM. Compared with the OM-specific receptor, the expression of the OM/LIF shared receptor is lower in both HME and breast cancer cells. Interestingly, neither the HME cells nor the breast cancer cells were growth-inhibited by LIF. In fact, LIF slightly stimulated the proliferation of breast cancer cells. These data suggest that OM and LIF have different effects on mammary epithelial cells and that the effects of OM are mediated by the OM-specific receptor. Taken together, these studies suggest that OM may function as a negative growth regulator for mammary epithelial cells through the interaction with the OM-specific receptor. The decreased or lost expression of this receptor, particularly the signaling subunit (OSMRβ), would result in an imbalance of the growth-stimulatory and growth-inhibitory regulation exerted in the microenvironment of breast tissue. This may be one of the factors contributing to the development of breast tumors.

To extend the these studies and to estabolish the possible roles of OM in human breast malignancy, we have focused our investigations into three different aspects. 1. to examine the effect of OM on the expression of breast cancer specific genes which play critical roles in tumor promotion, 2. to examine the signaling transduction pathways that lead to inhibition of tumor cell growth, and 3. to examine the role of endogenous OM, produced by the host defense system (T cells and macrophages) in breast cancer development by immunohistochemistry.

In this report, we show that: (1) The breast cancer specific gene 1 (BCSG1) is highly expressed in infiltrating breast carcinom but not expressed in normal breast tissue. (2) The expression of BCSG1 is transcriptional suppressed by OM. (3). The antagonism of OM to EGF mitogenic activity is mediated in part by inhibiting EGF-induced cellular tyrosine phosphorylation event. (4) Detection of OM in tissue samples of breast carcinoma and ovarian carcinoma.

B. MATERIALS AND METHODS

Cells and Reagents. The human breast cancer cell line H3922 was developed from a ductal infiltrating breast carcinoma at the Bristol-Myers Squibb Pharmaceutical Research Institute-Seattle. The cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human recombinant OM was expressed by Chinese hamster ovary cells and purified as previously described (22). The other growth factors and cytokines were obtained from R&D Systems, Minneapolis, MN. The plasmid containing the cDNA probe for *c-Myc* was obtained from ATCC. A human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) cDNA probe was generously provided by Dr. Jeff L. Ellsworth at CV Therapeutics, Palo Alto, CA.

Northern Blot Analysis

Total cellular RNA was isolated by the method of Peppel and Baglioni. Approximately 20 μg of each total RNA sample was separated on a 1.0% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described. The blot was hybridized at 60° C to a 0.55 Kb ^{32}P-labeled human *BCSG1* cDNA probe. The probe was labeled using 50 μCi [α-^{32}P] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN). The membrane was then washed 3 times at ambient temperature with 2X SSC, 0.1% SDS and twice at 37° C with 0.1X SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 1-3 days at -80° C. The *c-Myc* and *GAPDH* probes were prepared by random-primer labeling as described for the *BCSG1* probe. All other steps in analysis of the membrane were also followed as described for the *BCSG1* probe. The autoradiographs were scanned by a laser densitometer (Personal Densitometer^{TM} SI, Molecular Dynamics, Sunnyvale, CA) and the integrated intensity of each band was analyzed with the program ImageQuaNT^{TM}, version 4.1. Densitometric analysis of autoradiographs in these studies as well as those discussed below included various exposure times to ensure linearity of signals.

Nuclear Run-on Analysis. These analyses were conducted using a procedure adapted from one that had already been described. Briefly, 1.8 x 10^7 adherent H3922 cells were harvested with cell scrapers into a minimal volume of cold phosphate buffered saline (PBS). The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei samples were immediately frozen under liquid nitrogen and stored at -80°C.

The frozen nuclei were subsequently thawed and 100 μl of each sample received 100 μl 2X reaction buffer (70% glycerol, 0.02 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 μCi/μl [^{32}P] rUTP). The reactions were incubated, with shaking at 30°C for 30 minutes. Labeled nuclei were

pelleted and resuspended with 100 µl DNase buffer (50% glycerol, 20 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 minutes. Samples were brought up to 125 µl with 7.5 µl 13.6 mg/ml proteinase K, 5 µl 10 mg/ml yeast tRNA, and 12.5 µl 10X SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris-HCl, pH 7.4) and incubated at 42°C for 30 minutes.

Labeled RNA transcripts were extracted by adding the following: 275 µl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M b-mercaptoethanol], 45 µl 2.0 M sodium acetate, 450 µl water-saturated phenol, and 90 µl chloroform:isoamyl alcohol (49:1). The samples were vortexed and incubated on ice for 15 minutes. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples were dissolved with 102 µl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 2.0×10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham Life Sciences, Arlington Heights, IL) slot blot. Each blot received the following three plasmids: 5 µg plasmid with the human *GAPDH* cDNA insert, 3 µg of the 0.3 kb fragment of the *BCSG1* cDNA which is the 3' end of the cDNA. This fragment was generated by cutting the *BCSG1* cDNA with the restriction endonuclease BstX1. Probing the *GAPDH* plasmid allowed normalization of the *BCSG1* signals measured by densitometry.

Actinomycin D/mRNA Stability Analysis. H3922 cells in 100 mm tissue culture plates were incubated with or without OM for 6 h. Actinomycin D (5 µg/ml) was added to cells for different lengths of time. At the end of each time point, total RNA was harvested as described above under "Northern Blot Analysis". Electrophoresis of total RNA samples, blotting, and hybridization to radiolabeled probes were also carried out as described above.

Cell Proliferation Assay

Cells were seeded in 96-well tissue culture plates (Costar, Cambridge, MA) in IMDM medium containing 2% FBS at a density of 3000 cells/well in 100 µl of medium. Three to 5 hours after seeding, 50 µl of the same culture media containing various factors was added to each well. Three days later [^{3}H]thymidine (0.5 µCi/50 µl/well) in medium was added to the culture plates 4 hours prior to harvest. The amount of [^{3}H]thymidine incorporated into cells was measured using a liquid scintillation counter (Pharmacia, Piscataway, NJ). The differences in counts/min incorporated between experimental and control cultures were used as an index for DNA synthesis. Each data point represents the average of triplicate cultures and each experiment was performed at least 3 times.

Immunoprecipitation and Immunoblotting. H3922 cells were cultured in 60 mm culture plates for 2 days in 2% FBS IMDM with or without OM (100 ng/ml). The cells were then stimulated with 10 ng/ml of EGF for 10 minutes, or 100 ng/ml of OM for 15 minutes. Cells were rinsed with cold PBS and lysed with 0.5 ml of lysis buffer (50 mM Tris [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF, 5 µg/ml of aprotinin, 1 µg/ml of leupeptin, and 1.25 µg/ml of pepstatin). The cell lysate (250 µl, approximately 1µg/µl of protein) was

precleared with 50 µl of protein A-sepharose slurry and then incubated overnight with 30 µl of sepharose-conjugated anti-phosphotyrosine monoclonal antibody 4G10. The sepharose beads were collected by microcentrifugation and resuspended in 50 µl of 2X SDS sample buffer containing 5% β-mercaptoethanol. Immunoprecipitates were analyzed by western blot with anti-phosphotyrosine monoclonal antibody 4G10 using an enhanced chemiluminescence (ECL) detection system.

C. RESULTS

1. Transcriptional regulation of breast cancer specific gene (BCSG1) by oncostatin M

Northern blot analysis of BCSG1 expression in human breast cancer cell lines.

By utilizing a high-throughput direct-differential cDNA sequencing approach, a novel breast cancer specific gene, designated *BCSG1* was recently isolated from a breast tumor cDNA library (15). *BCSG1* gene is transcripted into a 1 kb mRNA, and the open reading frame of the full length gene is predicted to encode a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with genetic data base reveals that *BCSG1* is highly homologous to the non-Aβ fragment of human AD amyloid protein with 54% sequence identity (16).

BCSG1 mRNA expression was found exclusively in neoplastic epithelial cells. In situ hybridization analysis has demonstrated a stage-specific expression pattern of *BCSG1* mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade in situ breast carcinoma, to high expression in advanced infiltrating carcinomas. This implies that *BCSG1* may play a role in breast cancer malignant progression. The high level expression of BCSG1 in the neoplastic breast epithelial cells suggest that the expression of BCSG1 may be up-regulated in the mammary gland during breast cancer onset and progression.

Therefore, we initiate a study to examine BCSG1 expression in breast cancer cell lines. As shown in Figure 1, among the 8 different cell lines, BCSG1 mRNA was detected in 2/4 human breast cancer cell lines derived from pleural effusion and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (ZR-75-1, MDA-MB-231, H3914, and H3922). In contrast, no hybridization signals were detected from mRNAs isolated from 4 normal breast tissues.

Dose- and time-dependence of OM-induced suppression of *BCSG1* mRNA expression in breast cancer cells.

Among the cell lines that were examined, H3922 expressed the highest level of *BCSG1* mRNA. Since OM has an inhibitory and differentiative effect on H3922 cell growth, we examined the effect of OM on *BCSG1* mRNA expression. The results in Figure 2 demonstrated a dramatic time-dependent suppression of *BCSG1* mRNA level by OM. Treatment of H3922 cells with OM initiated an immediate decrease of *BCSG1* mRNA as early as 30 minutes. By 4 h treatment, the level of *BCSG1* mRNA was decreased to 70% of that in control, and by 24 h, the mRNA was completely undetectable (Figure 2).

We next investigated the dose-dependent effect of OM on *BCSG1* gene expression. In order to be able to detect *BCSG1* mRNA in OM treated cells, H3922 cells were treated with OM for 6 h at different concentrations before the total RNA was harvested for northern blot analysis of *BCSG1* mRNA (Figure 3A). Densitometry analysis of *BCSG1* hybridization signal with normalization to *GADPH* signal (Figure 3B) showed that cells treated with 0.2, 1.0, 5.0 , 25, and 125 ng/ml OM expressed 58%, 46%, 47%, 45%, and 41% as much *BCSG1* mRNA, respectively, as that observed in control cells treated with OM dilution buffer (1 mg/ml BSA in PBS). This data suggested that OM produced a maximum suppressive effect on *BCSG1* expression at a concentration of 1-5 ng/ml with an Ec50 of 0.08 ng/ml. To compare the effective dose ranges of OM in its ability to elicit two different biological responses (cell growth and

BCSG1 gene expression), in parallel experiment, the OM-dose dependent effect on H3922 cell proliferation was also examined. As shown in Figure 3C, the maximal inhibitory effect of OM on H3922 DNA synthesis occurred at OM concentration of 20 ng/ ml with an Ec50 of 0.2-0.4 ng/ml.

Transcriptional regulation of *BCSG1* expression by oncostatin M.

To determine whether the down regulation of *BCSG1* expression by OM occurs at transcriptional or post-transcriptional levels, we conducted nuclear run-on assays to measure the relative transcription rate of *BCSG1* in control cells and in the cells treated with OM. As shown in Figure 4, upon the pretreatment with OM for 16 h, H3922 cells contained only 28.5% as many active *BCSG1* mRNA transcripts as that observed in control cells. Data were normalized by the signals observed in the *GADPH* slots. The level of reduction of BCSG1 transcripts is consistent with the results obtained from northern blot. These results suggest that transcriptional regulation is the major component of the observed OM-mediated suppression of *BCSG1* gene expression.

To further investigate the mechanisms by which OM inhibits the expression of the *BCSG1* gene in H3922 cells, *BCSG1* mRNA stability was examined. Control cells and the cells treated with OM for 6 h were exposed to actinomycin D. Total RNAs isolated from the cells at various actinomycin D exposure time points were subjected to northern blot analyses of *BCSG1*. (Figure 5). Although OM treatment reduced the level of *BCSG1* mRNA to approximately 50% of that in control cells, the *BCSG1* mRNA levels in both the control cells and the OM-treated cells were not decreased by actinomycin D. In contrast, the *c-Myc* mRNA levels were rapidly declined with the treatment of actinomycin D. These data suggest that the *BCSG1* mRNA stability was not altered by OM, and that the *BCSG1* mRNA is relatively stable. An attempt to treat cells with actinomycin D for a longer period of time was not successful due to the actinomycin D-mediated toxicity in H3922 cells. These results together with the data generated from the nuclear run-on assay suggest that the *BCSG1* gene expression was suppressed by OM at the transcriptional level.

***BCSG1* gene expression was not down regulated by OM-related cytokines.**

Previous studies conducted in our laboratory have shown that the growth-inhibitory activity of OM in the breast cancer H3922 cells is a unique function of this cytokine. Several OM-related cytokines including IL-6, IL-11, and LIF did not inhibit the cellular proliferation of H3922 cells and other breast cancer cell lines such as MCF-7. We were interested in investigating whether these factors regulate BCSG1 gene expression. H3922 cells were treated with OM, IL-6, IL-11, and LIF for 24 h at 100 ng/ml concentration for each factor respectively. The result of northern blot analysis show that BCSG1 mRNA expression was specifically regulated by OM, but not by the other cytokines (Figure 6). These data suggest that the down regulation of BCSG1 expression is concurrently associated with the suppression of cell growth.

2. Suppression of EGF-mediated tyrosine phosphorylation by pretreatment of breast cancer cells with oncostatin M

Previously, we have shown that stimulation of H3922 cells with EGF increases cellular proliferation to 3-4-fold higher than that observed in untreated cells. Simultaneous treatment of H3922 cells with OM causes a time- and dose-dependent antagonistic effect on EGF-stimulated

growth. In order to investigate the impact of OM on EGF-mediated signal transduction, we examined EGF-induced tyrosine phosphorylation events in H3922 cells that had been pretreated with OM for different lengths of times. Tyrosine phosphorylated proteins were immunoprecipitated from total cell lysates derived from H3922 cells treated under various conditions. Subsequent western blot analysis demonstrated that EGF stimulation transciently induces tyrosine phosphorylation of several cellular proteins including the EGF receptors (EGFR). Pretreatment of H3922 cells with OM for 6 to 48 hours prior to EGF stimulation did not affect tyrosine phosphorylation. However, 72 hours pretreatment of cells with OM severely diminished the tyrosine phosphorylation events induced by EGF (Figure 7). We also observed that the maximal inhibitory effect of OM on H3922 cell proliferation occurs after three days treatment. We are currently investigating the mechanism by which OM affacts the cellular tyrosine phosphorylation event and the correlation between cell proliferation and tyrosine phosphorylation.

3. Detections of endogenous expressed OM in tumor tissue section.

One characteristic of the host response to tumor growth is the infiltration of tumors by macrophages and T lymphocytes. Production of tumor-inhibitory cytokines in a timely and locally released fashion may represent an important function of the host defense system to suppress tumor progression. From this prospective view, the inhibition of breast tumor growth by OM, a cytokine predominantly produced by activated T cells and macrophages may represent the tumor-host interaction. To examine this hypothesis, we have initiated a study to examine the OM expression in breast tumors by inflamatory cells. By utilizing anti-OM monoclonal antibodies, we examined OM expression in tissue sections derived from breast cancer and ovaian cancers. The preliminary results showed infiltrating macrophages and neutraphiles in breast tumors were positive with anti-OM mAbs. (Figure 8). This immunostaining signals were blocked with addition of recombinant human OM in the incubation medium. Interestingly, high levels of anti-OM immunoreactivity were detected in mucinous epithelial cells in ovarian tumors (Figure 9). The expression of OM are associated with the low malignant potential of this type of ovarian tumor. Whether the high level expression of OM in ovarian tumors plays a functional role in mucinous epithelial cells transformation need to be further investigated. We currently in the process to conduct in situ hybridization to examine the mRNA expression of OM in breast and ovarian tumor sections.

D. CONCLUSION

The results described in this report demonstrate that

1) BCSG1 gene expression is associated with breast cancer development and progression.

2) BCSG1 is transcriptional suppressed by OM in a breast cancer cell line which is growth-inhibited by OM. That suggests BCSG1 expression may be involved in the process of OM-mediated growth inhibition.

3) In OM treated H3922 cells, EGF no longer be able to induce the EGF receptor intrinsic tyrosine kinase activity. This may account for at least in part the mechanism by which OM antagonizes the EGF mitogenic activity.

4) The expression of OM by inflammatory cells that were infiltrating breast tumors suggest that OM may plays a functional role in vivo as a part of body defence system to inhibit tumor growth and progression.

E. REFERENCES

1. Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lioubin, M.N., and Todaro, G.J. 1986. Oncostatin M: A growth regulator produced by differentiated lymphoma cells. *Proc. Natl. Acad. Sci. USA*. 83:9739-9743.
2. Brown, T.J., Lioubin, M.N., and Marquardt, H. 1987. Purification and characterization of cytostatic lymphokines produced by activated human T-lymphocytes: Synergistic antiproliferative activity of transforming growth factor β1, interferon gamma, and oncostatin M for human melanoma cells. *J. Immunol.* 139:2977-2983.
3. Grove, R.I., Mazzucco, C., Allegretto, N., Kiener, PA., Spitalny, G., Radka, S.F., Shoyab, M., Antonaccio, M., and Warr, G.A. 1991. Macrophage-derived factors increase low-density lipoprotein uptake and receptor number in cultured human liver cells. *J. Lipid. Res.* 32:1889-1897.
4. Grove, R.I, Eberhardt, C., Abid, S., Mazzucco, C., Liu, J., Todaro, G., Kiener, P., and Shoyab, M. 1993. Oncostatin M is a mitogen for rabbit vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA*. 90:823-827.
5. Zhang, X.G., Gu, J.J., Lu, Z.Y., Yasukawa, K., Yancopoulos, G.D., Turner, K., Shoyab, M., Taga, T., Kishimoto, T., Bataille, R., and Klein, B. 1994. Ciliary neurotropic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J. Exp. Med.* 179:1343-1347.
6. Horn, D., Fitzpatrick, W.C., Gompper, P.T., Ochs, V., Bolton-Hansen, M., Zarling, J.M., Malik, N., Todaro, G.J., and Linsley, P.S. 1990. Regulation of cell growth by recombinant oncostatin M. *Growth Factors. 2*: 157-165.
7. Liu, J., Clegg, H.C., and Shoyab, M. 1992. Regulation of EGR-1, c-jun, and c-myc gene expression by oncostatin M. *Cell Growth. Differ.* 3:307-313.
8. Gearing, D.P., and Bruce, A.G. Oncostatin M binds the high-affinity leukemia inhibitory factor. 1991. *New Biol.* 4:61-65.
9. Gearing, D.P., Comeau, M.R., Friend, D.J., Gimpel, S., Thut, C.J., McGourty, J., Brasher, K.K., Kinh, J.A., Gillis, S., Mosley, B., Ziegler, S.F., and Cosman, D. 1992. The IL-6 signal transducer, gp130: An oncostatin M receptor and affinity converter for the LIF receptor. *Science. 255*:1434-1437.
10. Liu, J., Modrell, B., Aruffo, A., Marken, J.S., Taga, T., Yasukawa, K., Murakami, M., Kishimoto, T., and Shoyab, M. 1992. Interleukin-6 signal transducer gp130 mediates oncostatin M signaling. *J. Biol. Chem., 267*: 16763-16766.
11. Pennica. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J. Biol. Chem.* 270:10915-10922.
12. Liu, J., Modrell, B., Aroffo, A., Scharnowske, S., and Shoyab, M. 1994. Interactions between Oncostatin M and the IL-6 signal transducer, gp130. *Cytokine*. 6:272-278.
13. Mosley, B., Delmus, C., Friend, D., Thoma, B., and Cosman, D. 1994. The oncostatin M specific receptor: cloning of a novel subunit related to the LIF receptor. *Cytokine*. 6:539. (Abstr.)
14. Mosley, B., lmus, C.D., Friend, D., Boian, N., Thoma, B., Park, L., and Cosman, D. Dual oncostatin M (OSM) receptors: cloning and characterization of an alternative signaling

subunit conferring OSM-specific receptor activation. J. Biol. Chem. *271*:32635-32643, 1996

15. Ji, H., Liu, Y.E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B.K., Rosen, C., and Shi, Y.E. Identification of a breast cancer-specific gene, *BCSG1*, by direct differential cDNA sequencing. Cancer Research, *57*:759-764, 1997.

16. Ueda, K., Fukushima, H., Masliah, E., Y., Iwai, A, Yoshimoto, M., Otero, D.A., Kondo, J., Ihara, Y., and Saitch, T. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. Proc. Natl. Acad. Sci. USA, *90*: 11282-11286, 1993.

Figure 1

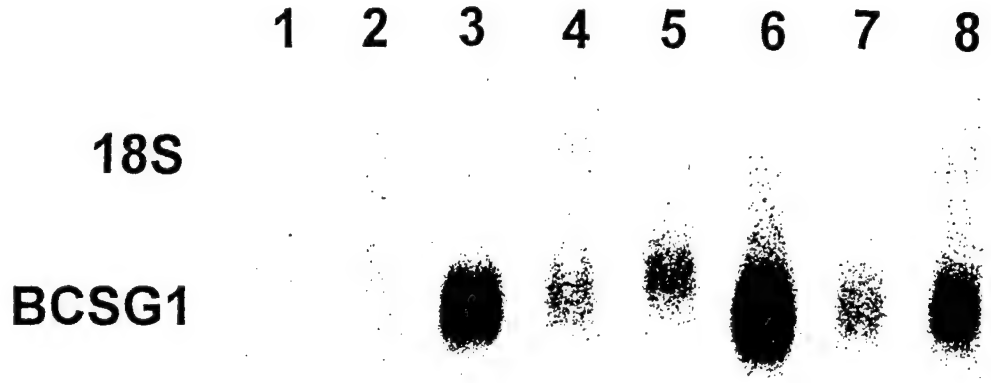


Figure 2

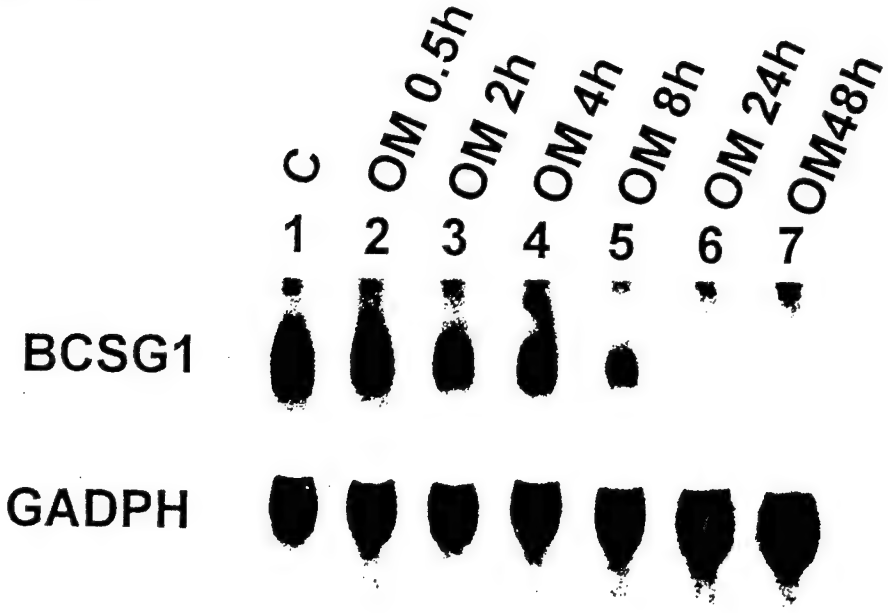


Figure 3A

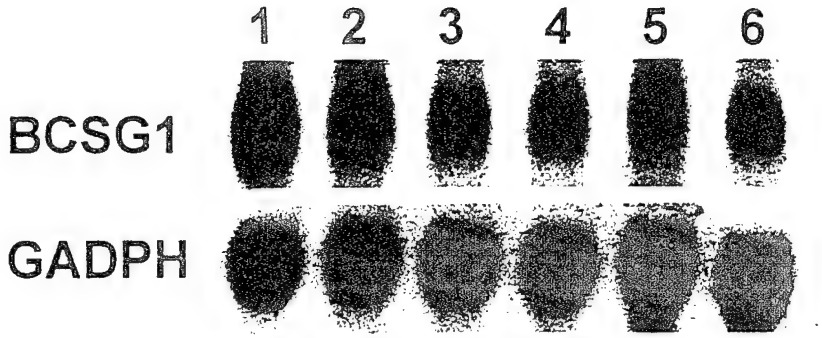


Figure 4

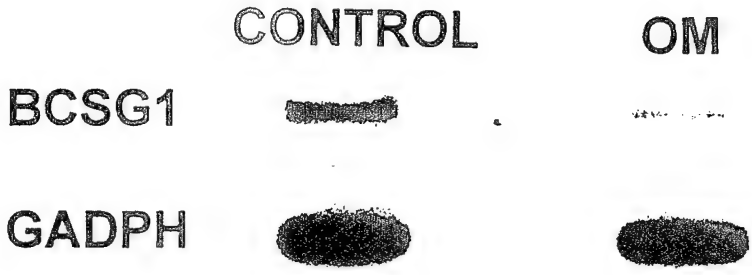


Figure 3B

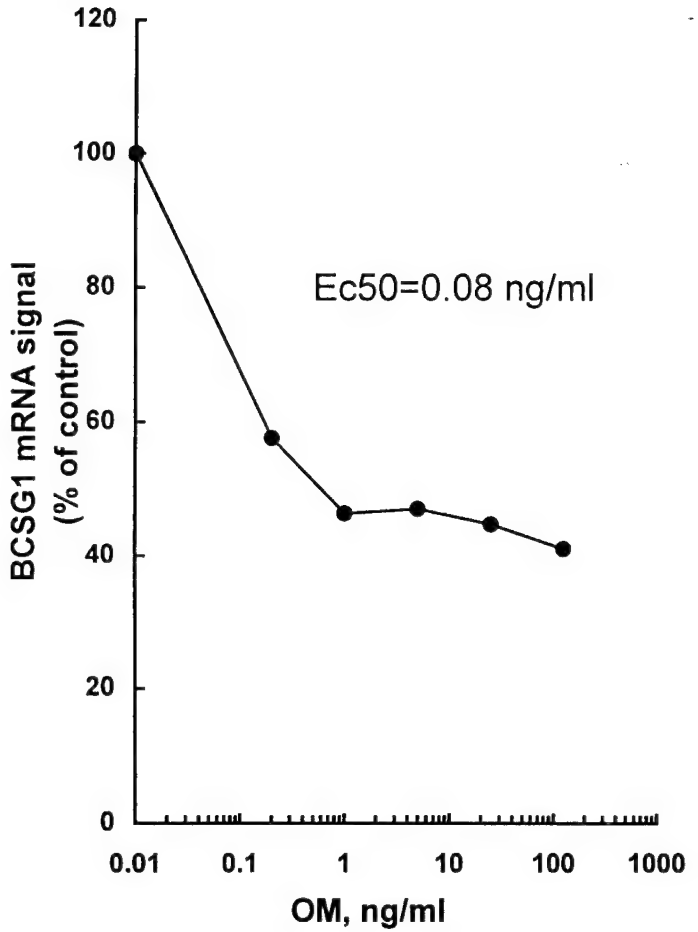


Figure 3C

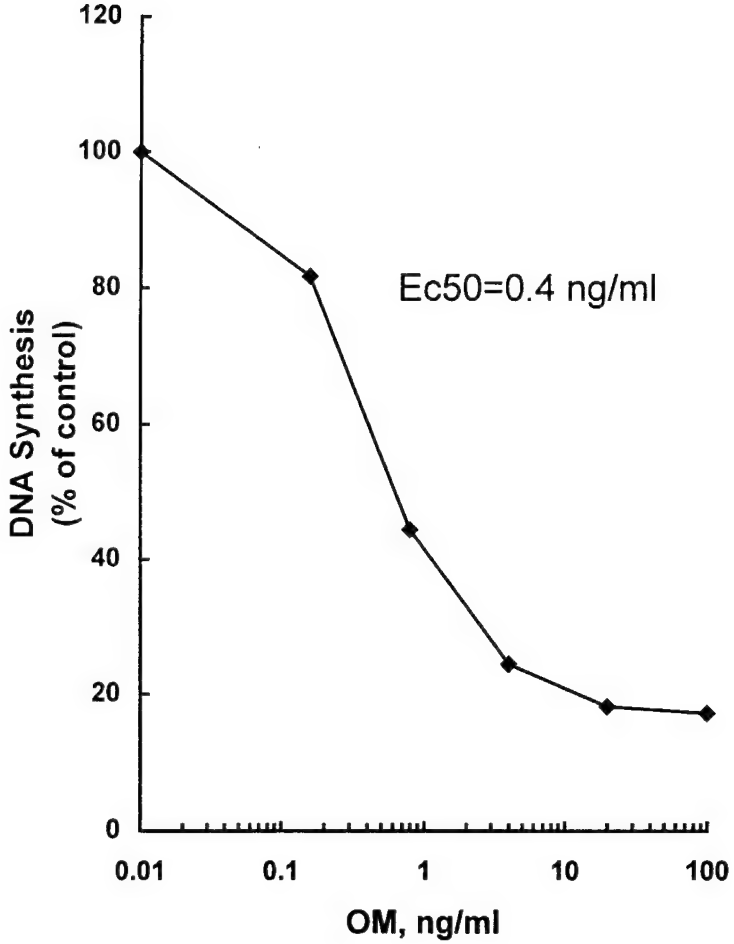


Figure 5

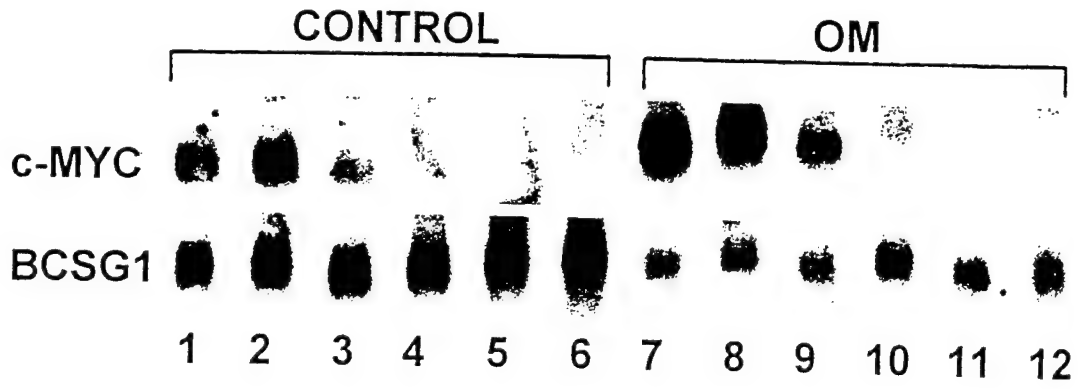


Figure 6

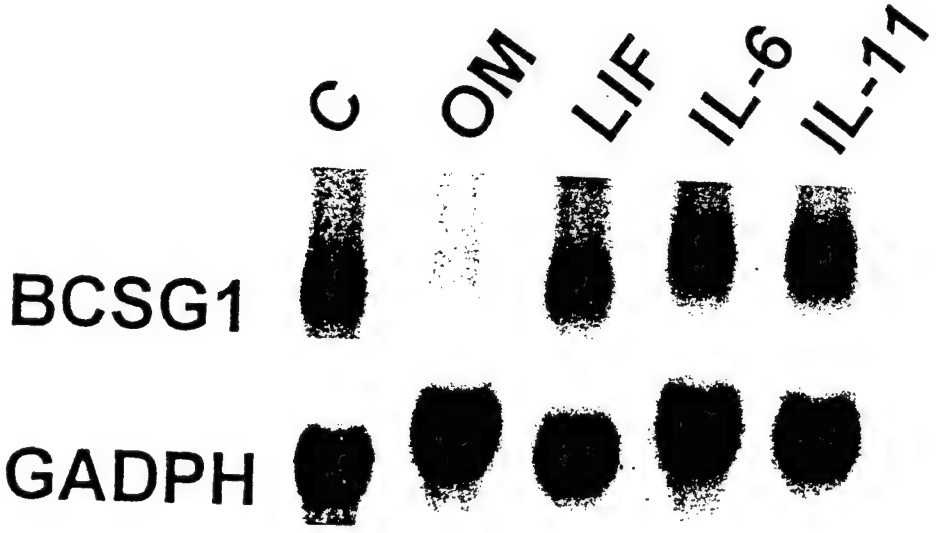


Figure 7

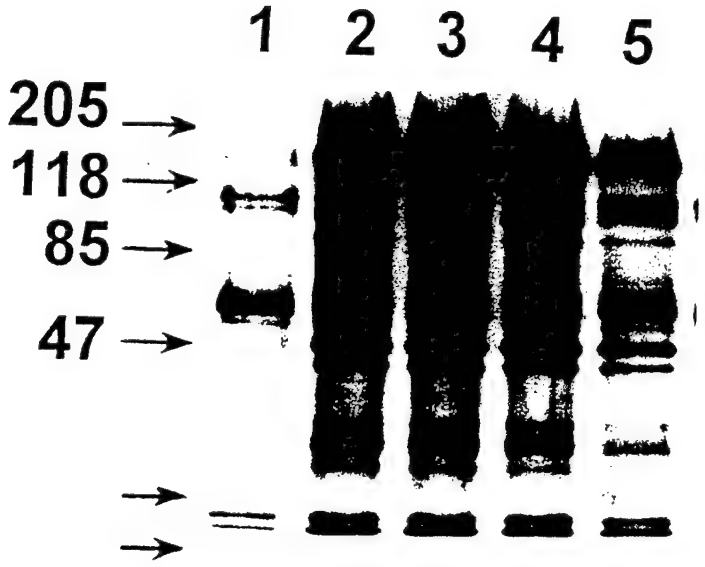
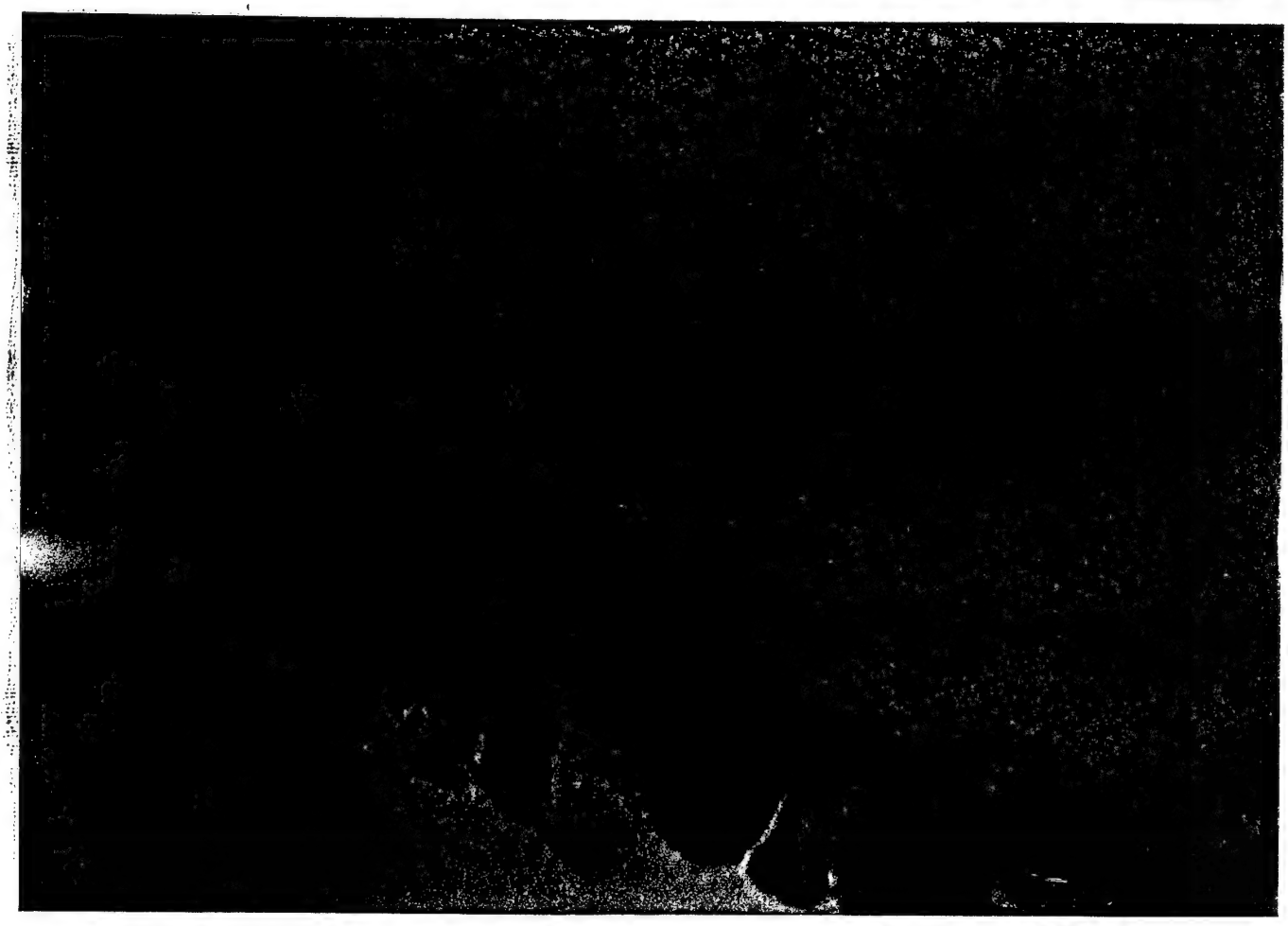


Figure 8

Figure 9



G. FIGURE LEGENDS

Figure 1. Northen blot analysis of BCSG1 expression in human breast cancer cell lines. Total RNA was isolated and analyzed by northern blot. Lane 1, H3396 (derived from pleural effusion); Lane 2, MCF-7 (derived from pleural effusion); Lane 3, SKBR-3 (pleural effusion); lane 4, MDAMB-231 (pleural effusion); Lane 5, H3914 (infiltrating ductal carcinoma); lane 6, H3922 (infiltrating ductal carcinoma); Lane 7, ZR-75-1 (infiltrating ductal carcinoma); Lane 8, T47D (infiltrating ductal carcinoma).

Figure 2. Time-dependent suppression of *BCSG1* mRNA expression by OM. Total RNA (20 μg/lane) was isolated from H3922 cells that were cultured in 2% FBS IMEM and treated with OM at a dose of 20 ng/ml for the indicated lengths of time. RNA samples were blotted onto a nylon membrane and hybridized to a ^{32}P-labeled 0.55 Kb *BCSG1* cDNA probe as described in "Materials and Methods". The blot was rehybridized under the same conditions with a ^{32}P-labeled human *GAPDH* probe. Radioactive signals were detected by autoradiography and quantified by densitometry.

Figure 3. OM-concentration dependent effects on *BCSG1* mRNA expression and on cellular proliferation.
A. Total RNA (20 μg/lane) was isolated from H3922 cells that were untreated (lane 1), 0.2 ng/ml OM-treated (lane 2), 1.0 ng/ml OM-treated (lane 3), 5.0 ng/ml OM-treated (lane 4), 25 ng/ml OM-treated (lane 5), or 125.0 ng/ml OM-treated (lane 6). All OM treatments were for 6 h. RNA samples were analyzed as described in figure 1. B. The relative levels of *BCSG1* mRNA normalized to *GADPH* mRNA on the autoradiographam shown in panel A were quantitated by scanning densitometry. The figure shown is representative of two separate experiments. C. H3922 cells (3000 cells/well) were incubated for 72 hours in IMDM containing 2% FBS with the indicated amount of purified human recombinant OM. Cells were pulsed with [^{3}H]thymidine for an additional 4 hours. The amount of radioactivity incorporated into the cells was determined by scintillation assay and the data were expressed as percentages of that observed in the untreated controls.

Figure 4. Nuclear runon analysis of *BCSG1* transcription. Two slots were blotted onto each of two nylon membrane strips. One slot received 3 μg of the 0.3 kb fragment of the *BCSG1* cDNA which is the 3' end of the cDNA. This fragment was generated by cutting the *BCSG1* cDNA with the restriction endonuclease BstX1. The second slot was loaded with 5 μg of the *GAPDH* plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear runon reaction prepared from 16-h OM-treated H3922 cells. The second was hybridized to a labeled nuclear runon reaction prepared from control cells. Equal amount of radioactivity was used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. Isolation of nuclei, preparation of nuclear runon reactions, hybridizations and washes were all as described in "Materials and Methods".

Figure 5. OM did not change the *BCSG1* mRNA stability. Cells were either treated with OM at the concentration of 50 ng/ml for 6 h (lane 7-12), or treated with OM dilution buffer, 1 mg/ml

BSA in PBS, for the same length of time (lane 1-6), then actinomycin D at a concentration of 5 μg/ml was added to cells for different lengths of time. At the end of each time point, total RNA was harvested and analyzed for *BCSG1* mRNA and *c-Myc* mRNA expressions as described in Figure 1. The length of actinomycin D treatment: lanes 1 and 7, 0 h; lanes 2 and 8, 0.5 h; lanes 3 and 9, 1 h; lanes 4 and 10, 2 h; lanes 5 and 11, 3 h; lanes 6 and 12, 4 h.

Figure 6. Comparison of effects of OM related cytokines on *BCSG1* gene transcription. H3922 cells were treated for 24 h with each factors at 100 ng/ml concentration respectively. Total RNA was subsequently isolated for northern blot analysis of *BCSG1* mRNA levels.

Figure 7. Oncostatin M has an antagonistic effect on EGF-mediated tyrosine phosphorylation of signaling proteins in H3922 cells. Tyrosine phosphorylated proteins immunoprecipitated from various H3922 cell extracts were detected on a western blot from an SDS-PAGE gel by enhanced chemiluminescence. Lane 1, untreated control cells; Lane 2, OM stimulated ten minutes; Lane 3, EGF stimulated ten minutes; Lane 4, both OM and EGF stimulated ten minutes; Lane 5, OM pretreated 72 hours, followed by EGF stimulation ten minutes. Ten-minute stimulation of H3922 cells with EGF induced tyrosine phosphorylation of many proteins (EGF 10′). Preteatment of H3922 cells with OM for 72 hours prior to stimulation with EGF (OM72h./EGF10′), reduced the number of detectable tyrosine phoshphorylated signals nearly to that observed in the control lane (C). These data demonstrated a clear antagonistic effect of OM on EGF-activated signaling components in H3922 cells.

Figure 8. Immunohistochemical staining with anti-OM mAbs in breast tumor sections. OM staining was detected in neutraphil cells. In breast tumor section, the malignant epithelial cells were negative.

Figure 9. OM positive staining in mucinous epithelial cells in ovarian tumors.

H. PUBLICATIONS (5/1996-4/1997)

1. Liu J, Wallace PM, Forcier K, HellstrÖm I, Vestal RE: Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth, antagonism of growth factors, and down regulation of c-myc protooncogene. *Cell Growth & Differentiation,June, 1997.*
2. Spence M, Vestal RE, and Liu J: Suppression of c-myc proto oncogene by oncostatin M in breast cancer cells. *Cancer Researc, June, 1997..*
3. Shi YE, Wang M, Jia T, Liu J, Fuchs A, Rosen C, Liu YE, and Ji H: Identification of breast cancer specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Researc* 57:759-764, 1997.
4. Liu J, Streiff R, Vestal E, and Briggs M: Novel Mechanism of Transcriptional Activation of Hepatic LDL Receptor by Oncostatin M, *J. Lipid. Res in press.*
5. Liu J, Zhang YL, Spence MJ, Wallace PM, and Grass D: Liver LDL receptor mRNA in human ApoB and CETP double transgenic mice is down regulated by high-cholesterol diet and up regulated by cytokine oncostatin M. *Arteriosclerosis and Thrombosis in press.*
6. Liu J, Hadjokas N, Mosley B, Vestal RE: Oncostatin M-specific receptor expression and function in regulating cell proliferation of normal and malignant mammary epithelial cells. Submitted to Cytokines.
7. Liu J, Spence MJ Zhang YL, Jia T., Shi E: Transcriptional suppression of the breast cancer specific gene (*BCSG1*) expression by the cytokine oncostatin M. Submitted to Cancer Research.
8. Spence M, Vestal RE, and Liu J: Oncostatin M suppresses the EGF-induced intrinsic EGF receptor tyrosine phosphorylation activity.

I. AWARD

One of the two recipients for **Young Investigator Award**, International Cytokine Society, October 1996

Request for Immunex Research Materials

This Request must be completed and signed by the Investigator and an authorized representative of the Institution with which the Investigator is affiliated. The fully signed form must be returned by mail (**do not fax**) to Kathleen S. Picha, Immunex Corporation; 51 University Street, Seattle, WA 98101. Requests will be granted based on scientific merit, mutual interest and reagent availability.

Name of Investigator: Jingwen Liu, Ph.D.

Title or Function: Chief. Molecular pharmacology Research Laboratory

Name of Institution:
Veterans Affairs Medical Center

Office Address: Research Service, VA Medical Center
500 West Fort Street, Boise, ID 83702

Telephone Number: (208) 422-1000 ext. 7682 Facsimile Number: (208) 422-1155

E-mail: JingLiu@micron.net

Relevant Immunex contact(s): Bruce Mosley

Materials Requested (please specify reagent, species, and quantity or amount):
Human ~~recombinant~~ Oncostatin M receptor beta subunit cDNA in a mammalian expression vector (OSMR-beta)
in microgram quantity.

Address to which Materials are to be shipped: VA Medical Center
500 West Fort St. Boise. ID 83702

Federal Express Account Number or Credit Card Number and Expiration Date (to cover shipping costs only):
Federal Express Account #: 1424-9489-2

Address at which Materials will be stored:
Molecular pharmacology Research Laboratory. VA Medical Center
500 W. Fort St. Boise. ID 83702

Please describe proposed use of requested materials in one or two paragraphs:
We have been collaborating with Bruce Mosley to investigate the Oncostatin M receptor (OSMR) mediated growth inhibition of breast cancer cells. Several Breast cancer cell lines do not express OSMR and lack the response to OSM-mediated growth regression. Therefore, we would like to transfect OSMR-beta cDNA into these cells and to test their response to OSM.

Keywords (please mark all that apply)

[ ] AIDS models	[ ] Dendritic Cell Biology	[ ] Immunodeficiency	[ ] Neurobiology	[ ] Tumor Immunology
[ ] Adjuvants	[ ] Developmental Biology	[ ] Infectious Disease	[ ] Pharmacology	[ ] TURF
[ ] Antigen Presentation	[ ] ECCAT	[ ] Inflammation	[ ] Reproductive Biology	[ ] Urinary System Biology
[ ] Autoimmunity	[X] Endocrinology	[ ] Joint/Connective Tissue Biology	[ ] Respiratory Biology	[ ] Vascular Biology
[ ] B cell Biology	[ ] Gastrointestinal Biology		[X] Signal Transduction	[ ] Virology
[ ] Bone Biology	[ ] Gene Therapy	[ ] Mast Cell Biology	[ ] Skin Biology	[ ] Other (please specify)
[ ] Cardiac Biology	[ ] Genetic Disorders	[ ] Metabolism	[ ] T cell Biology	[ ]
[ ] Cell Adhesion	[ ] HIV/SIV	[ ] Muscle Biology	[ ] Tissue Repair	[ ]
[ ] Cell Death	[ ] Hematopoiesis Biology	[ ] NK cell Biology	[ ] Transplantation	[ ]
[ ] Cytokine Induction	[ ] Hypersensitivity	[ ] Neoplasia	[ ] Tumor Drug Resistance	[ ]

Questions concerning this Agreement should be directed to Immunex Extramural Research (206) 389-4026.

LIU - 23 ABT

Immunex Materials Transfer Agreement

You (the "Investigator") have requested that Immunex supply certain Materials that are identified in the accompanying Request for Immunex Research Materials ("Request"). The Materials requested are considered by Immunex to be highly valuable, confidential and proprietary products of Immunex research. Immunex is willing to provide the Investigator with reasonable quantities of the Materials upon the following terms:

1. Definitions. "Materials" means all materials transferred by Immunex to the Investigator hereunder, any replicates, progeny and derivatives of the Materials, and any mixtures or combinations of Materials and other substances. "Proposed Use" shall mean any use reasonably consistent with the Brief Statement of Proposed Use of requested materials set forth in the Request.

2. Title to Materials. Immunex shall retain all title and interest in and to the Materials. The Investigator shall not imply or represent to any person that he/she is the owner of the Materials.

3. Delivery of Materials. Immunex shall deliver the requested Materials to the Investigator within a reasonable period of time after this Agreement is fully-executed by the parties. Immunex shall determine the quantities to be transferred to the Investigator.

4. Use of Materials. As of the date this Agreement is executed by Immunex, Immunex grants the Investigator a non-exclusive license to use the Materials for the Proposed Use for academic research purposes only and not for any commercial use. The Investigator shall not use the Materials in humans or in contact with any cell or other materials to be infused into humans. Use of the Materials to prepare somatic cell therapy products or gene therapy products is specifically prohibited. The Investigator shall use the Materials in compliance with all applicable federal, state and local laws and regulations. The Investigator shall not transfer the Materials to any person who is not under the immediate and direct supervision of the Investigator, nor use the Materials in research that is subject to consulting or licensing obligations to another corporation or a government agency, other than the NIH, unless prior written permission is obtained from Immunex.

5. Reports. At least every six (6) months, the Investigator shall provide Immunex with a written report summarizing the status or results of the Proposed Use of the Materials. The Investigator or Institution shall provide Immunex with a copy of any manuscript or other proposed publication at least thirty (30) days before it is submitted for publication and shall inform Immunex of the content of any proposed oral disclosure at least thirty (30) days prior to such disclosure. The Investigator or Institution shall delay submission of any publication, at the request of Immunex, for up to an additional ninety (90) days after receipt by Immunex of the proposed publication, in order to permit filing of appropriate patent applications. Immunex shall hold all such communications in confidence. The Investigator and Institution agree that any data developed under this Agreement may be used by Immunex to support Immunex patent applications or in preparing regulatory filings, without any compensation to the Investigator or Institution.

6. Inventions. If the use of the Materials hereunder results in an invention or discovery including a new use, improvement, or enhancement of the Materials, whether patentable or not, ("Invention") the Investigator or Institution shall disclose the Invention to Immunex. The Investigator and Institution shall cooperate with Immunex in seeking patent coverage for the Invention. As consideration for the receipt of the Materials from Immunex, Investigator and Institution shall grant Immunex a royalty-bearing, worldwide exclusive license, with the right to sublicense, to all of the Investigator's and Institution's rights in the Invention. If a law or government regulation prohibits Investigator or Institution from granting Immunex such exclusive license, Investigator and Institution shall grant Immunex a license having exclusivity terms that are as favorable to Immunex as permissible under such laws or regulations. The Investigator, Institution and Immunex shall negotiate the terms of such license to Immunex in good faith. The royalty for such license shall be in the range of one to three percent (1% to 3%) based on the net sales of any Invention. Factors to be considered in determining such royalty are: the commercial potential for the Invention in the marketplace, the existence of competing products or technologies, the proprietary position of the Invention, the likelihood and timing of any regulatory approval, the investment required to commercialize the Invention, the respective contribution of the parties to the Invention as well as any other relevant factors. The parties shall define such net sales in accordance with customary commercial practices at the time they set the royalty rate within the range established herein.

7. Expression Vectors. If the Materials include an expression vector, the Investigator and Institution shall use the vector for the Proposed Use only, and not for any other use. The Investigator or Institution shall not use the vector to isolate or express cDNA clones not included in the Materials. If the Institution or Investigator fails to comply with the terms of this paragraph 7, the Institution or Investigator agree to assign to Immunex all of their right, title and interest in and to such cDNA clones.

8. No Conflicts. The Investigator represents that he or she is authorized to enter into this Agreement and that no other contract or other obligation conflicts with the obligations to be assumed by the Investigator under this Agreement.

9. Term and Termination. This Agreement shall be effective as of the last date signed by the parties below and shall terminate two (2) years thereafter. This Agreement may be earlier terminated at any time by any party upon ten (10) days' written notice. All unused Materials shall be returned to Immunex or destroyed, at the sole option of Immunex, within ten (10) days following termination of this Agreement. Termination shall not affect any rights of any party under paragraphs 5, 6 or 10.

10. Disclaimer. THE MATERIALS ARE PROVIDED TO THE INVESTIGATOR WITHOUT WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. IMMUNEX SHALL NOT BE LIABLE FOR ANY USE OF THE MATERIALS BY THE INVESTIGATOR OR THE INSTITUTION, OR FOR ANY LOSS, CLAIM, DAMAGE, OR LIABILITY, OF ANY KIND OR NATURE, WHICH MAY ARISE FROM OR IN CONNECTION WITH THIS AGREEMENT OR FROM THE USE, HANDLING OR STORAGE OF THE MATERIALS. No indemnification for any loss, claim, damage, or liability is intended or shall be provided by any party under this Agreement.

11. Assignability. The rights and obligations of the Investigator and the Institution under this Agreement shall not be assignable without the prior written consent of Immunex.

12. Amendments. No modification of this Agreement shall be effective unless the modification is in writing and signed by an authorized representative of each party.

13. Applicable Law. This Agreement and the rights of the parties shall be determined in accordance with the laws of the State of Washington. In the event of actual or threatened disclosure or transfer of the Materials by the Investigator to a third party without the prior written consent of Immunex, Immunex is likely to suffer irreparable harm, and shall be entitled to specific performance of the obligations of the Investigator and Institution under this Agreement, without bond, as well as all necessary injunctive relief against unauthorized disclosure or transfer.

The signatures of the authorized officers of each party are required below to make the Agreement effective.

IMMUNEX CORPORATION

Michael B. Widmer

April 4, 1997

Michael B. Widmer, Ph.D.
Vice President, Director of Biological Sciences

Date

The provisions of the foregoing are agreed to and accepted by:

Jingwen Liu, ph.D

4/7/97

Investigator

Date

VA Medical Center
Boise, ID

Name of Institution

Authorized Representative of Institution

Medical Center Director

4-7-97

Title

Date

Rev. 1/97